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One of the most common alterations in human breast cancer is the overexpression of the c-erbB-2 proto-oncogene that occurs in 25-30% of human breast tumors. Recently, *in vitro* studies have shown that this increased expression correlates with the presence of the transcription factor AP-2. We have therefore begun to determine the role of AP-2 both in normal mammary gland development and in breast cancer. An analysis of human breast tumor samples reveals that there is a significant increase in AP-2 protein expression in the progression from benign breast epithelium. Moreover, the presence of various AP-2 family members correlates with the expression of important signal transduction molecules including c-erbB-2, ER and IGF-IR. We have also found that the AP-2 genes are differentially regulated during mouse mammary gland development. We are now directly testing the role of AP-2 in breast development and breast cancer using animal model systems. Mice containing expressed transgenes for both wild-type and dominant negative versions of AP-2 have been generated. Analysis of these mice reveals that AP-2 exerts a profound influence over the growth and morphogenesis of the mammary gland.

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Introduction

Breast cancer is one of the most common and important diseases affecting women in North America and Western Europe (1, 3). Despite advances in treatment, only modest increases in survival have been achieved. Examination of the molecular changes in human breast cancer has revealed that one of the most common alterations is the overexpression of the normal c-erbB-2 gene. The human c-erbB-2 gene, which is normally expressed at low levels in a variety of adult epithelial cells, is overexpressed in 25-30% of carcinomas of the breast (2). Moreover, high levels of c-erbB-2 expression have been shown to correlate with poor prognosis and to predict a worse response to therapy (2, 3).

The normal c-erbB-2 gene (also known as *neu* or HER2) encodes a 185 kDa tyrosine kinase transmembrane receptor which shows extensive structural similarity with the epidermal growth factor receptor (17, 18). The relationship between c-erbB-2 and tumorigenesis was first noted when this gene was identified as the causative agent of chemically induced rat neuroglioblastomas (19). This rat oncogene contained a single base pair mutation, resulting in an amino acid change in the transmembrane domain of the protein. This alteration leads to autophosphorylation of the cytoplasmic portion of the protein which renders c-erbB-2 constitutively active and oncogenic (19, 20). This change is unlikely to occur in the human population because of codon usage differences; the same amino acid change in the human gene would require the unlikely occurrence of mutations in two adjacent base pairs. In fact, in a number of human tumors examined so far no equivalent mutation to the rat activated *neu* gene has been found (21-23). Instead, the involvement of c-erbB-2 in human cancers always seems to involve a different mechanism, namely the overexpression of the normal gene (see below).

The potential of the c-erbB-2 gene to cause tumors has been assessed in both cultured cells (4, 5) and transgenic mice (6, 7). In tissue culture it has been shown that the normal human c-erbB-2 cDNA was able to induce transformation of NIH3T3 cells when fused to strong viral transcriptional control elements. The transgenic mouse experiments were first performed using the oncogenic version of the rat c-erbB-2 gene, *neu*, placed under the control of the mouse mammary tumor virus (MMTV) LTR to direct its expression to breast tissue. These transgenic mice gave rise to adenocarcinomas encompassing the entire mammary gland with extremely short latency (6). More recently, these experiments have been recapitulated using the normal version of the rodent *neu* gene under the control of the MMTV LTR. Transgenic mice carrying this construct developed focal mammary tumors after long latency periods that metastasized at high frequency (7). Thus, inappropriate overexpression of the normal *neu* gene in mammary tissue can induce breast carcinoma, supporting a role for this gene in human disease.

One of the fundamental questions arising from these studies is what is the mechanism of c-erbB-2 overexpression in human mammary carcinomas? In general the increase in c-erbB-2 mRNA and protein is associated with amplification of the gene (2),

although it can also occur from a single copy gene (8, 9,16). Indeed, studies in cell lines derived from human mammary tumors have shown that there is a 6-8 fold increase of c-erbB-2 mRNA per template copy in overexpressing cells, whether or not the gene is amplified (8, 9). The critical region of the human c-erbB-2 promoter that is responsible for the activation in overexpressing mammary cancer cells has recently been identified (11). Transfection and DNaseI footprinting assays have led to the identification of a transcription factor, originally termed OB2-1, present in c-erbB-2 overexpressing cells, that binds and activates the c-erbB-2 promoter (10). More recent studies involving our laboratory have shown that OB2-1 is antigenetically and functionally indistinguishable from the developmentally regulated transcription factor AP-2 (11-14).

The 50 kDa AP-2 protein was initially isolated from human Hela cells by virtue of its interaction with an important GC-rich recognition sequence present in the SV40 and human metallothionein IIa (hMtIIa) enhancers (24, 26). A structure function analysis indicated that AP-2 is a sequence-specific DNA binding transcription factor with a modular organization (13, 14). The N-terminus of the protein contains a region rich in proline and aromatic amino acid residues that serves as an activation domain both in the context of AP-2 or when attached to a heterologous DNA binding domain. The C-terminal half of AP-2 is responsible for sequence-specific DNA binding. This DNA binding portion consists of a basic region necessary for DNA contact and a large, stable dimerization domain of novel structure, termed a helix-span-helix (13, 14). AP-2 can bind to *cis*-regulatory sequences present in a variety of viral and cellular genes, including proenkephalin, collagenase, TNFα, growth hormone, HTLV-1, and MHC class I H-2K^b (24-28, 33). More recent reports have also implicated AP-2 in the transcriptional control of the estrogen receptor, hsp27, epithelial cadherin and p21$^{waf1/cip1}$ (35-36, 49, 53).

In the context of c-erbB-2, the critical promoter sequence required for high level expression in mammary tumor cell lines occurs approximately 200 nts upstream of the major RNA start site (10). This sequence, GCTGCAGGC, is closely related to the AP-2 consensus sequence, GCCNNNGGC (13). Functional assays confirmed that AP-2 was able to bind to this sequence and regulate c-erbB-2 expression in mammary derived cell lines (11). In co-transfection assays, the wild type AP-2 protein was able to stimulate c-erbB-2 promoter driven expression in mammary cells lacking AP-2. In contrast, a dominant negative version of AP-2 that only contained the dimerization domain was able to reduce expression in cell lines containing endogenous AP-2 protein. Finally, tumor cell lines containing high levels of c-erbB-2 were found to have high levels of AP-2 protein (11). Taken together, these studies strongly suggest that AP-2 may play an important role in human breast cancer.

Further examination of the OB2-1 complex indicated that it contained other proteins in addition to AP-2. In particular, peptide sequence derived from OB2-1 demonstrated that it is actually a mixture of three proteins belonging to the AP-2 gene family (37). In addition to original AP-2 gene, two novel AP-2 genes present in the OB2-1 complex have been isolated and characterized. A comparison of the predicted peptide sequence reveals that these new genes, AP-2 β and AP-2 γ, are highly related to the

original AP-2 protein, now termed AP-2 α. The greatest degree of conservation is observed in the regions of the protein originally shown to be important for DNA binding and transcriptional activity. In particular, the DNA binding and dimerization domains are very well conserved (75-85%). Consistent with this finding, the three AP-2 proteins all recognize the same DNA sequence and can form either homo- or hetero-dimers with themselves or with each other (37). Furthermore, these three proteins are all capable of activating expression of reporter genes driven by the human c-erbB-2 promoter (37). Taken together, these data indicate that the c-erbB-2 gene can act as a target for transactivation by AP-2 proteins.

We and others have recently established the critical importance of AP-2α as a developmental regulator in murine embryogenesis. The AP-2α–null-mice have severe defects in many organs including the head, brain, peripheral nerves, limbs, and the ventral body wall - where the mammary gland normally resides (38, 39). In contrast, little is known about the role of AP-2 in adult mouse development. Because AP-2 is a fundamental gene regulator and is associated with breast cancer, we have begun to determine the role of AP-2 both in normal development of mammary gland and in breast cancer. My specific aims were to use transgenic technology to examine the role of AP-2 in normal breast development and to test its potential to cause mammary cancer. Since July 1, 1997, I have made significant progress in pursuit of these specific aims. In particular, as outlined in more detail below, I have determined the pattern of AP-2 expression during normal growth and development of the mouse mammary gland. In addition, in collaboration with investigators at Yale School of Medicine, I have found that a high incidence of AP-2 gene expression in human breast cancer correlated with the regulation of multiple growth factor pathways. I have also established breeding colonies of several AP-2 transgenic mouse lines. More recently, I have quantitated transgene expression in these lines, and have begun an analysis of their altered mammary gland phenotypes.

Materials and Methods

Tissue Collection and Histological Analysis

Mouse mammary gland tissue samples were obtained by removing the fourth inguinal gland from mice at various stages of postnatal development, i.e. from virgin, pregnant, lactating and regressing mice. The tissue specimens were fixed with 10% natural buffered formalin, dehydrated in a graded series of ethanol and xylene, then embedded in paraffin wax. Next, 6 μm sections were cut and mounted on poly-L-lysine coated slides. These sections were stained with hematoxylin and eosin or used for immunohistochemical analysis. Whole-mount analysis of mouse mammary tissue was performed as previously described (50). Human breast cancer biopsies were obtained from Yale School of Medicine. These biopsies have already been analyzed in terms of patient history, including such criteria as age, tumor recurrence, and response to therapy. The biopsies were either embedded in OCT medium (Bayers Inc) or in paraffin wax. A protocol for the study was approved by the Human Investigations Committee at the Yale University School of Medicine.

Antibodies and Immunohisochemistry

Details for antibodies and procedure for immunohistochemistry have been reported in the annual report of 1997.

Construction of Transgenes

Transgenes were constructed using standard subcloning techniques. A FLAG epitope tag was attached to the 3′ end of the AP-2 cDNA. This tag does not affect the function of the AP-2 protein as a DNA binding transcription factor (T. Williams, unpublished) and enables us to distinguish between expression of the transgene and endogenous gene using an antibody specific for this novel FLAG epitope (available from Kodak). The fusion constructs of wild type or dominant negative forms of AP-2 were then placed under control of mouse mammary tumor virus long terminal repeat (MMTV LTR) using the plasmid pMSG (Pharmacia) which also contains a 3′ SV40 polyadenylation signal. The rat neu cDNA was placed under control of the human c-erbB-2 promoter. The constructs were digested with appropriate restriction enzymes to remove vector sequences since those sequences are commonly believed to interfere with the transgene expression (29-31). The transgenes were then separated from the vectors by 10-40% sucrose density gradient ultracentrifugation. The fractions that contained transgene fragments were collected and dialyzed extensively against 10 mM Tris.Cl, 0.25 mM EDTA, pH7.5, and their concentration was adjusted to 1-3 μg/ml for microinjection.

Generation of Transgenic Mice

Transgenic mice were generated by injecting DNA into pronuclei of fertilized eggs of inbred FVB mice (Taconic). The embryos surviving the microinjection were transferred into oviducts of pseudopregnant females (CD1 strain, Charles River). After birth, mice carrying the transgene were identified by Southern blotting using genomic DNA isolated from tail biopsies. The expression of transgenes was determined by RNase protection.

Isolation of Genomic DNA and Southern Blot Analysis

Genomic DNA was isolated from tails of 4-5 weeks old mice as described (40). 12 μg of genomic DNA was digested with appropriate restriction enzymes, electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose (Schleicher & Schuell) or Hybond N filters (Amersham). The filters were hybridized with probes derived from the transgenes which were either radioactively labeled by random-priming or labeled nonradioactively with "Genius Nonradioactive Nucleic Acid Labeling and detection Kit" (Boehringer-Mannheim). The hybridized products were visualized by autoradiography or by light-emission respectively.

Isolation of RNA and RNase Protection

Mouse mammary tissue was homogenized in guanidine isothiocyanate buffer and the total RNA was isolated as described (41). RNase protection was performed essentially as described (12), except that 10 μg of total RNA was used for each protection assay.

Results

Correlation between Incidence of AP-2 Gene Expression and the Regulation of Multiple Growth Factor Pathways in Human Breast Cancer

Previous *in vitro* studies have indicated that the AP-2 family of proteins may be involved in the etiology of human breast cancer. Critical AP-2 binding sites are present in both the c-erbB-2 and estrogen receptor promoters and the AP-2 genes are expressed in many breast cancer cell lines (11, 53). We have now extended these *in vitro* studies to examine the expression of the AP-2 proteins in primary human breast cancer tissue. For these experiments, specific immunological reagents were developed which enable the presence of the AP-2α and AP-2γ family members to be detected in archival tissue sections (34).

Data obtained from this analysis demonstrated that of the 74 tumors analyzed, 65 (88%) contained at least one of the AP-2 proteins and only 9/74 tumors (12%) lacked appreciable levels of the AP-2 proteins (34). Strikingly, there was a large increase in the expression of AP-2γ in the breast cancers in comparison to benign breast epithelia ($p=0.01$). We were also able to correlate the AP-2 data with c-erbB-2 protein levels since many of these patient samples were previously examined for c-erbB-2 expression (52). Appreciable levels of c-erbB-2 were observed in 7/69 tumors. All of the c-erbB-2 positive tumors expressed AP-2. The most striking correlation, however, was that c-erbB-2 expression was almost entirely restricted to tumors which expressed both AP-2α and AP-2γ. Of the 38 tumors which expressed either AP-2α or AP-2γ alone, only one had high levels of c-erbB-2. In marked contrast, 6/24 (25%) AP-2α /AP-2γ double positive tumors expressed c-erbB-2 ($p=0.003$) (34). These findings strongly support previous *in vitro* evidence which indicated that AP-2 may be an important transcriptional regulator of c-erbB-2.

The data obtained for AP-2 expression status were also analyzed in terms of the estrogen receptor (ER) and progesterone receptor (PR) positivity of the tumors. These studies revealed that there was also a significant correlation between AP-2α expression status and ER positivity ($p=0.018$) (34). Since an AP-2 binding site has been identified in the ER promoter (53), our findings support a role for AP-2 in the regulation of ER transcription. The correlations found between AP-2, c-erbB-2 and ER expression prompted us to examine another signalling molecule that has been implicated in breast cancer - namely the insulin-like growth factor I receptor (IGF-IR). Overexpression of the IGF-IR may predict for poor survival characteristics in breast cancer patients, and the receptor has been demonstrated to mediate an anti-apoptotic phenotype (70). Significantly, we found that elevated levels of IGF-IR expression did not occur in the absence of AP-2γ ($p=0.04$). Taken together, our findings strongly support a role for the AP-2 gene family in the control of cell growth and differentiation in breast cancer.

Expression of Endogenous AP-2 in Mouse Mammary Gland

In parallel with my studies on the expression of AP-2 in human breast cancer, I have been examining the pattern of AP-2 expression during normal growth and maturation of the mammary gland. We have examined a limited number of human benign breast epithelia samples, but the majority of my studies have focused on the mouse mammary gland for the following two reasons. First, the mouse provides an experimental system in which samples representing the complete range of mammary gland development can be readily obtained for analysis. Second, understanding the normal pattern of AP-2 expression in the mouse mammary gland is an integral component of the transgenic experiments in which I am targeting ectopic expression of AP-2 to this organ.

In the annual report of 1997, I reported preliminary experiments in which antibodies specific for each of the AP-2 proteins had been characterized for their ability to work on mouse mammary gland tissue. My data demonstrated that AP-2 proteins were readily detected in the ductal epithelia of the virgin mouse mammary gland and also in the alveolar epithelial cells of pregnant mice. In the past year I have completed these immunohistochemical studies and complemented this analysis with RNase protection assays performed on adult mouse mammary tissue throughout mammary gland development. As shown in Figure 1, both AP-2 α and AP-2 γ were expressed with a similar developmental profile. Transcripts from both genes were readily detected in the early virgin mammary tissue and expression levels increased as the mammary gland continued to mature. Higher levels of AP-2 expression were observed in pregnant mammary tissue. As gestation progressed, the expression of AP-2 continued to increase and reached a peak at 15 days of pregnancy, the latest pregnant mammary tissue analyzed. The expression was sharply reduced in lactating mammary tissue and reactivated during involution stage. The data are completely consistent with my immunohistochemical results and suggest that the AP-2 proteins have a role in regulating the growth and differentiation of mammary epithelia. In future, I will determine the correlation between the expression of AP-2, ER, PR and c-erbB-2 throughout various stages of development of mammary gland.

Detection of the Transgene Expression in the Transgenic Mouse Lines

As discussed above, AP-2 genes are differentially regulated during the normal development of the mouse mammary gland. We wish to further understand the role of AP-2 in the development of the mammary gland and also in tumorigenesis. Therefore, I am generating mouse model systems to determine the consequence of ectopically expressing AP-2 in the mouse mammary gland. In the previous report, I presented my preliminary characterization of several founder transgenic mice. I now report that I have successfully generated four types of transgenic mouse line which can transmit the respective transgene, namely MMTV/LTR driving wild-type AP-2α, MMTV/LTR driving the DNA binding domain of AP-2 α (ΔN165), MMTV/LTR driving the dimerization domain of AP-2α (ΔN278), and human c-erbB-2 promoter driving rat *neu*

(c-erbB-2). (N. B. The second and third types of transgene are dominant negative versions of AP-2).

I am now utilizing these transgenic mice to test the involvement of AP-2 in mammary gland development and to ascertain its potential to induce mammary tumors either alone or in combination with the *neu* transgene. In this regard I have begun an examination of the expression of the transgenes in the Southern positive lines. In these experiments, total RNA was isolated from mammary tissue of 10-14 day pregnant mice. RNase protection assays were performed on the RNA from 10 MMTV/LTR-wild-type AP-2α transgenic lines. Overexpression of the AP-2α transgene was noted in the mammary glands of six lines (Figure 2a). Three lines (MA44, MA7 and MA14) with higher expression levels were kept for further analysis. Lower amounts of transgene transcript were also detected in lung, salivary gland and kidney in these three lines. RNase protection was also performed on the RNA from six MMTV/LTR-ΔN278 dominant negative transgenic lines and two of these demonstrated overexpression of the transgene (Figure 2b). Similarly, one of two MMTV/LTR-ΔN165 dominant negative transgenic lines expressed this transgene (Figure 2c).

Effects of Overexpression of Wild-type AP-2α on the Development of Mouse Mammary Gland

Three distinct periods of mouse mammary gland development occur post-natally (15, 51). Ductal growth occurs from 6-8 weeks of age when epthelial end buds ramify throughout the fatty mesenchyme from the nipple to create a tree-like network of ducts. The onset of pregnancy initiates the second phase of development in which lobulo-alveolar structures develop from the existing ductal system. In the third phase, following weaning of young, the mammary gland undergoes extensive remodeling, leading to the loss of the alveolar structures, in a process involving large scale apoptosis. It should also be noted that in older virgin mice, 12-14 weeks of age or older, lateral branching also begins to occur and gradually leads to the formation of lobuloalveolar-like structures. However, complete development of lobuloalveolar structures occurs only during pregnancy and lactation.

In the mice with overexpression of AP-2α in the mammary gland, the ductal outgrowth through the fatty mesenchyme seemed not to be affected. By 8 weeks of age in the two transgenic lines so far analyzed the ductal network had filled the entire fat pad of the mammary tissue (Figure 3b) similar to their normal littermates (Figure 3a). In older virgin mice, however, there was a dramatic difference in lateral branching of the ducts. At the age of 6 months, the ductal system of normal littermates had a highly branched organization and formed lobuloalveolar-like structures (Figure 3c, e). In contrast, mammary glands from the transgenic mice displayed a simpler, sparser, ductal network, with a dramatic reduction both of side-branching and lobuloalveolar-like structures (Figure 3d, f).

Ductal development is a necessary prelude to lobuloalveolar formation. An examination of the transgenic mice demonstrated significant underdevelopment of the

lobuloalveolar structures during pregnancy consistent with the severe impairment of side-branching during ductal development. As shown in Figure 4a, c, by day 15 of pregnancy, the normal mammary gland has developed well-formed alveoli. In contrast, the transgenic gland had far fewer alveoli and these were less well-developed (Figure 4b, d). Histological examination of H & E stained sections, revealed fewer clusters of alveoli, and showed that each individual alveolus was smaller and less well expanded as compared to the normal littermate (Figure 4e, f). Moreover, lumen formation in the transgenic mice (f) was underdeveloped in comparison to their normal littermates (e).

The examination of the effects of the dominant negative transgenes on mammary gland development are in an earlier stage. Nevertheless, preliminary results suggest that these transgenes may induce overproliferation of the mammary epithelial network. This stands in marked contrast to the inhibition of ductal growth seen with overexpression of wild-type AP-2. A more thorough characterization of the dominant negative phenotype will be undertaken in the upcoming year.

Effects of Human c-erbB-2 Promoter/ Neu Transgene on the Development of the Mouse Mammary Gland

Though I am still in the process of characterizing the expression profile of the human c-erbB-2 promoter/rat neu transgene I have begun an analysis of the phenotype of these mice. An overgrowth of the mammary ductal network was observed in the virgin mammary gland of the transgenic mice, comparing Figure 5a (normal) and Figure 5c (transgenic). By crossing these transgenic mice with the mice overexpressing AP-2α, I obtained mice that were doubly transgenic. The analysis of these mice is ongoing, but preliminary studies suggest that the overexpression of AP-2α may inhibit overgrowth caused by *neu* gene, comparing Figure 5c (*neu* transgenic) with Figure 5d (doubly transgenic).

Discussion

Eukaryotic transcription factors are responsible for a variety of cellular processes during the development and maintenance of an organism. These proteins can form a regulatory cascade that is a fundamental mechanism for determination of cell fate. Because these molecules are such powerful regulators of genetic information, aberrant transcription factor activity can lead to developmental abnormalities and oncogenesis. Previous studies involving our laboratory and others have implicated AP-2 in the regulation of ER and c-erbB-2 expression in breast cancer (11, 37, 57). My studies, funded by the USAMRMC fellowship, have greatly strengthened the connection between AP-2 and human breast cancer and have also demonstrated that AP-2 can exert a profound influence on the growth and morphogenesis of the mammary gland.

One aspect of my research has been to examine the expression of the AP-2 proteins in human breast cancer. These studies demonstrated that there is an increased incidence of AP-2 protein expression during the progression from benign breast epithelia to breast cancer. Moreover, the presence AP-2 proteins correlates with the expression of potential targets of AP-2 which are important molecular markers of breast cancer. In particular, we found significant correlations between AP-2α and ER expression, and between AP-2γ and IGF-IR expression. We had also previously demonstrated several links between AP-2 and c-erbB-2 transcription *in vitro*. The data obtained from the analysis of human breast cancer biopsies reinforced the connection between AP-2 and c-erbB-2 expression, since no significant c-erbB-2 protein was detected in tumors lacking AP-2. Indeed, the majority of c-erbB-2 expression was limited to tumors containing both the AP-2α and AP-2γ proteins. One possible explanation for these correlations comes from the finding that AP-2α and AP-2γ genes can form functional heterodimers *in vitro* (37). Therefore, the correlation between c-erbB-2 expression of AP-2α/AP-2γ double positivity *in vivo* may indicate that there is a preference for heterodimers between these two transcription factors in the activation of c-erbB-2 transcription. Taken together, these data reinforce evidence linking the AP-2 transcription factor family to the regulation of cell:cell communication and cell growth in the developing mammary gland.

I wish to have a thorough understanding of the role of AP-2 in mammary gland development and mammary tumorigenesis. As a first step towards this goal, I have determined the expression of AP-2 throughout postnatal mouse mammary gland development using both immunohistochemistry and RNAse protection. My studies showed that the AP-2α and AP-2γ proteins are located in the ductal epithelial cells of virgin mice and the lobuloalveolar epithelial cells of pregnant mice. The expression levels of these AP-2 genes increase as the mice develop to maturity and reach a peak at late stages of pregnancy. The expression is down-regulated to a barely detectable level during lactation. These data suggest the following model for the role of AP-2 in mammary gland cell-fate determination. The stages of maturation when AP-2 is observed correspond to the timepoints when there is marked proliferation of the ductal network. Conversely, when the cells stop dividing and assume their terminal state of differentiation, no significant levels of AP-2 can be observed. Taken together, these

findings indicate that AP-2 may be involved in mammary gland proliferation and the early stages of differentiation into the lobuloalveolar network. Another interesting aspect of my analysis is that AP-2 is reactivated during involution. At this stage, the mammary gland undergoes extensive remodeling, leading to the loss of the alveolar structures, in a process involving large scale apoptosis (42, 43). In future, it will be interesting to determine if at this stage of mammary gland biology, AP-2 marks either the cells that are targeted to survive, or those destined to undergo apoptosis, or both. Thus, my experiments provide a basis for understanding the importance of AP-2 in growth, maturation and remodeling of mammary gland and will also help us understand the consequences of inappropriate expression of AP-2 both in the transgenic mice and also in tumorigenesis.

I have now generated transgenic mice which overexpress the wild-type AP-2α gene in the mammary gland. My studies demonstrate that overexpression of AP-2α under the control of the MMTV LTR results in an impairment of side-branching morphogenesis in virgin mice, and of lobuloalveolar development during pregnancy. Aberrant expression of several other genes can also impair mammary morphogenesis. For example, mice lacking either the inhibinβB (54) or the C/EBPβ genes (55, 56) have defects in ductal outgrowth, side branching, and development of lobuloalveolar structures. Mice with gene knockouts of cyclin D1 (57), stat5a (58), progesterone receptor (59), prolactin receptor (60), A-myb (61) and colony stimulating factor-1 (62) also have major defects in lobuloalveolar development. Analysis of the mammary glands of estrogen receptor knockout mice at 4 months of age revealed primitive ductal rudiments devoid of terminal end buds suggesting a role of this receptor in ductal outgrowth (63). Transgenic mouse studies have also revealed a number of genes that can influence mammary gland morphogenesis. Overexpression of TGF-β1 using either the MMTV promoter or the WAP promoter has been found to inhibit ductal proliferation or lobuloalveolar development, respectively (64, 65). Similarly, overexpression of whey acidic protein has also been reported to interfere with lobuloalveolar development during pregnancy and lactation (66). Finally, overexpression of parathyroid hormone-related protein (PTHRP) or parathyroid hormone also impaired branching morphogenesis during mammary gland development (50). I have begun a detailed histological comparison between the AP-2α transgenic animals and these other mouse models. My preliminary analysis suggests that the AP-2α phenotype is unique alhough at certain stages of mammary gland development it shares some similarity to the defects caused by the overexpression of either PTHRP and TGF-ß1. These studies help to indicate potential targets for AP-2 transcriptional control and will enable me to position AP-2α in the signalling network controlling mammary gland development.

The AP-2 proteins are a class of transcription factors that are thought to control genes involved in cell:cell contact, cell:cell signalling, and cell proliferation. My *in vivo* studies confirm that AP-2 can exert a profound influence on growth and morphogenesis. I have now begun to examine how the expression of potential AP-2 target genes are altered in the transgenic mice. So far I have not been able to detect expression of *neu* in MMTV/AP-2α singly and AP-2α/neu doubly transgenic mice. One

explanation for this finding is that *neu* levels are usually very low in the normal mammary gland. Alternatively, the impairment of mammary gland growth seen in the AP-2α transgenic mice may limit the amount of *neu* positive tissue that occurs in the mammary gland. The data obtained from the study of human breast cancer samples also suggests that AP-2α alone may not be sufficient to cause overexpression of the c-erb-2 gene. Instead, it may be necessary to overexpress both AP-2α and AP-2γ in the mammary gland to cause the overexpression of the c-erbB-2 gene, but these experiments are beyond the scope of the fellowship. In future, I will examine the expression of other potential downstream targets of AP-2, including the ER and IGF-IR. In addition, it will be of interest to determine if there is any connection between AP-2 and the other genes mentioned above which have inhibitory effect on the development of ductal and lobuloalveolar structures in the mammary gland.

My previous analysis of the AP-2α knockout mice has demonstrated that AP-2 is a critical regulator of many aspects of mammalian embryogenesis (38, 39). However, with respect to the mammary gland, the tissues which gives rise to the earliest stage of the fetal mammary gland are actually absent in these animals. Thus, it is not possible to determine the consequences of loss of AP-2α expression for mammary gland development using this model system. Fortunately, the AP-2 gene family offers a suitable target for an alternative dominant negative strategy to determine the consequences of removing this gene. I have therefore generated transgene constructs that will express dominant negative forms of the AP-2α protein, namely the DNA binding domain alone or the dimerization domain alone. So far, we have obtained two transgenic mouse lines with overexpression of the dimerization domain of AP-2α in the mammary gland, and one that overexpresses the DNA binding domain. Analysis of the effect of these dominant negative forms on the development of the mouse mammary gland is underway.

The data I have obtained from the study of AP-2 in human breast cancer and in the transgenic animal model system provides important new evidence pertinent to the role of AP-2 in breast cancer pathology. Previous evidence obtained *in vitro* concerning the role of AP-2 in cellular transformation suggested two alternative hypotheses for the presence of increased expression of AP-2 detected in breast cancer. One possibility was that the AP-2 proteins are proliferative signals which activate a variety of growth factor pathways. The inappropriate activation of the AP-2 proteins seen in breast cancer would thus drive ductal epithelial cells continually through the cell cycle. This theory would be supported by experiments in which overexpression of the AP-2α gene increased the oncogenic potential of PA-1 teratocarcinoma cells (32). An alternative hypothesis is that the AP-2 proteins are more akin to tumor suppressors, and the activation of the AP-2 genes would represent a failed attempt to halt cell proliferation. In this instance, the up-regulation of AP-2 target genes would occur as a secondary consequence of increased AP-2 levels. Several lines of evidence also support this theory. First, transfection of an AP-2α expression construct into HepG2 hepatocarcinoma cells or SW 480 adenocarcinoma cells can inhibit their growth and tumorigenicity (49). In these experiments, the observed decrease in cell growth correlated with an increase in the expression of the AP-2 target gene, $p21^{waf1/cip1}$, a

cyclin-dependent kinase inhibitor. Chromosomal mapping of AP-2α also supports a role of this gene as a potential tumor suppressor. The human AP-2α gene, *TFAP2A*, is located on chromosome 6p24, a region associated with loss of heterozygozity in several types of cancer, including follicolar center cell lymphoma, ovarian carcinoma, and head and neck squamous cell carcinoma (36, 67-69). My transgenic mouse studies, in which AP-2α is overexpressed in the mammary gland, also support the second hypothesis - that AP-2 may inhibit growth - since there is a marked inhibition of cell proliferation. In future, it will be important to determine if the expression levels of $p21^{waf1/cip1}$ are altered in the AP-2α transgenic mice. Indeed, further studies are clearly needed to determine what downstream targets are regulated by AP-2 and how the AP-2 genes are regulated in both normal development of mammary gland and tumorigenesis.

In conclusion, my data strongly support a role for AP-2 in controlling gene expression in both normal development of mammary gland and also in breast cancer. My further studies will provide a detailed mechanistic insight into the role of AP-2 in mammary gland development and the etiology of breast cancer.

The Experiments to Be Done in Final Year of the Fellowship Period

In the final year I will perform following studies.

1). Analyze the phenotype of mice with overexpression of AP-2α in more detail. This will include analyzing the phenotype of lactating and regressing mice, and studying more timepoints for virgin and pregnant mice.

2). Analyze the phenotype of the mammary gland in mice which overexpress the dominant negative form of AP-2α. I will study various timepoints during mammary gland maturation including virgin, pregnant, lactating and regressing.

3). Characterize the molecular mechanisms responsible for the phenotypes of transgenic mice which overexpress either the wild-type or dominant negative versions of AP-2. In particular, I will examine known targets of AP-2, such as ER, IGF-IR, c-erbB-2 and $p21^{waf1/cip1}$. Furthermore, I will analyze how other molecular markers of mammary gland cell fate determination are expressed in the transgenic mammary glands. These studies will involve RNAse protection and immunohistochemical analysis.

4). Given the phenotypes of the wild-type and dominant negative transgenic mice we predict that AP-2 will modify the incidence of tumorigenesis in the established mouse models of breast cancer. We have obtained preliminary evidence that the incidence of AP-2 expression in human breast cancer correlates with p53 status (T. Williams, unpublished observation). Therefore, we will cross the MMTV/AP-2α mice to a p53+/- or -/- mice to determine how AP-2 influences tumor incidence in the absence of a functional p53 gene.

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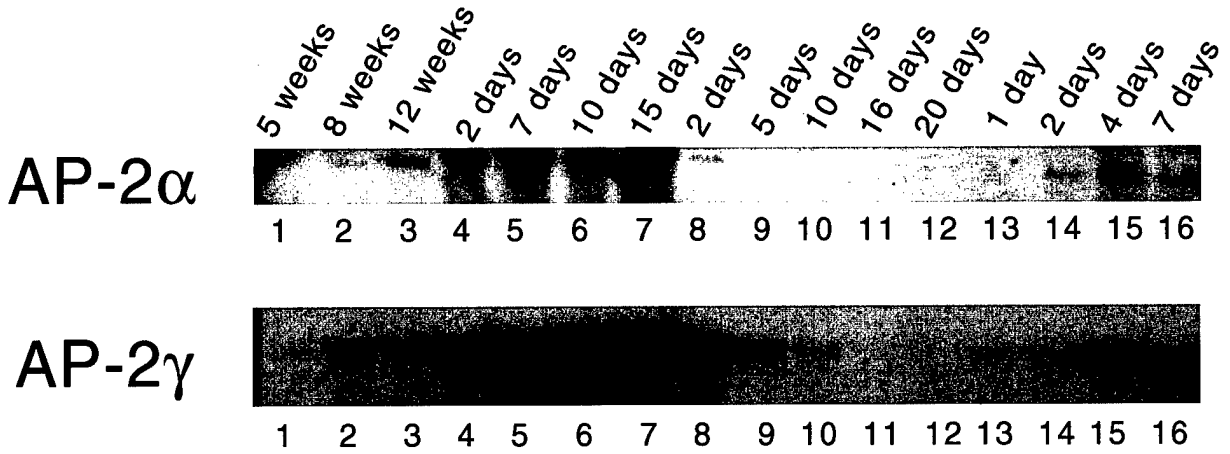

Figure 1. Expression of endogenous AP-2 in mouse mammary gland. RNase protection assays were performed on mouse mammary tissue through different developmental stages. Mammary tissues were taken from virgin mice (Lanes 1-3); pregnant mice (Lanes 4-7); lactating mice (Lanes 8-12) and regressing mice (Lanes 13-16) at the timepoints shown on the top of the figure.

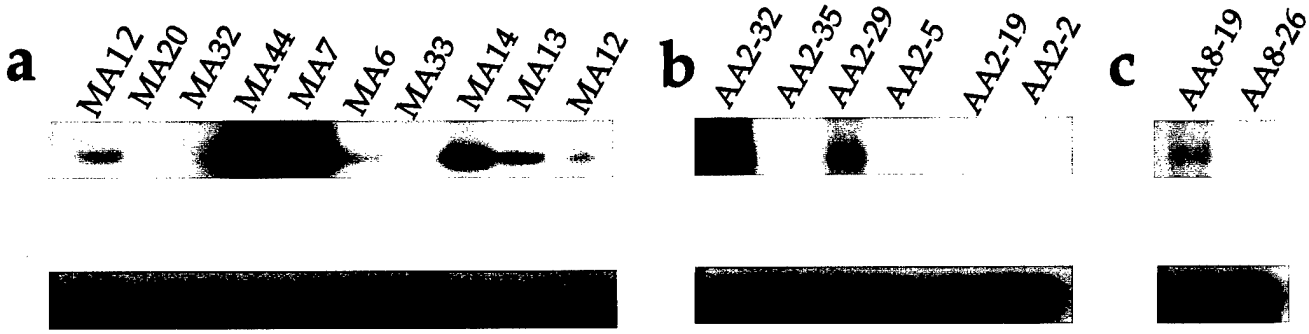

Figure 2. RNase protection analysis of 10 μg of total cellular RNA prepared from 14-day pregnant mammary tissues of MMTV/wild-type AP-2α (a) or MMTV/dominant-negative form (b, c) transgenic mice. The top bands represent the expression of transgene from various transgenic mouse lines while the bottom bands correspond to mouse actin mRNA as a loading control. The wild-type transgene was expressed in transgenic mouse lines MA12, MA44, MA7, MA14, MA13 and MA12. The dimerization domain transgene was expressed in transgenic mouse linesAA2-32 and AA2-29 and DNA binding domain transgene was expressed in line AA8-19.

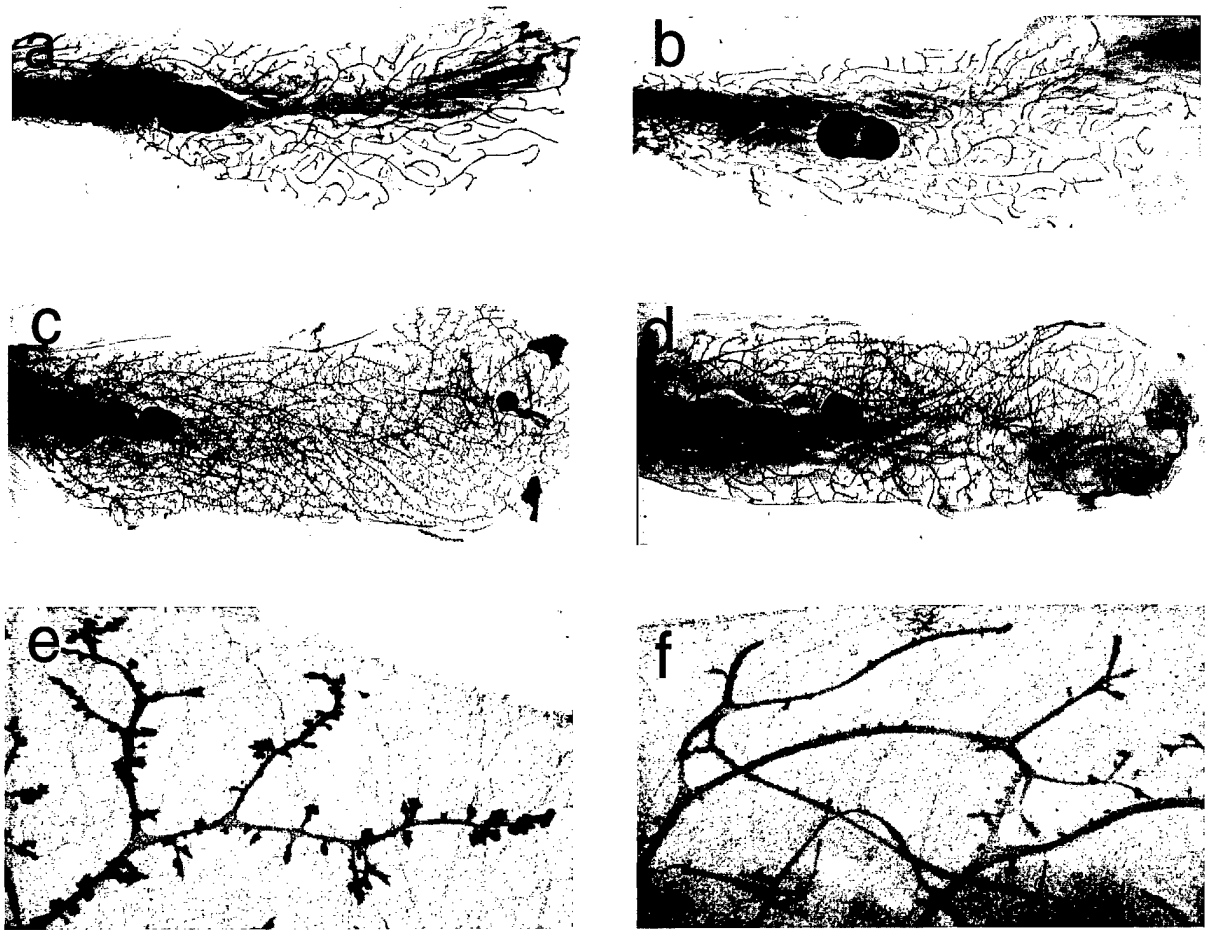

Figure 3. Whole-mount analysis of the 4th inguinal mammary gland taken from transgenic (b, d, f) or normal littermate (a, c, e) virgin mice. (a, b) 8 week-old mice. (c, d, e, f) 6 month-old mice. The dark oval in the center of the glands is a lymph node. (e, f) Higher magnifications of the periphery of the glands shown in (c) and (d). Note that the transgenic ducts have fewer side branches and lack of lobuloalveolar-like structures (f) as compared with the normal gland (e).

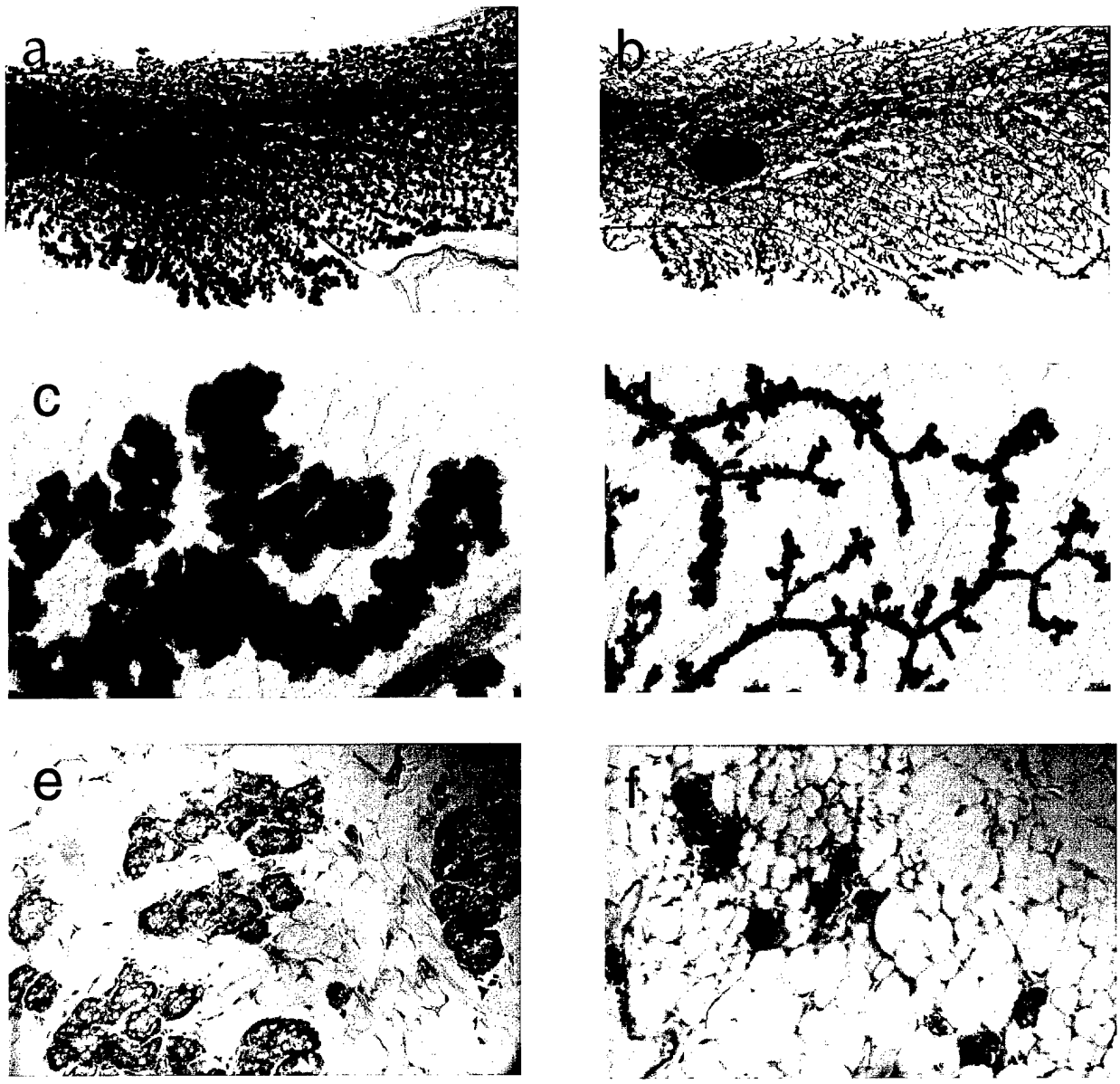

Figure 4. Analysis of the 4th inguinal mammary glands taken from transgenic (b, d, f) and normal littermate (a, c, e) mice at 15-day pregnancy. (c, d) Higher magnification of the periphery of the glands shown in (a) and (b). (e, f) Conventional sections (6 μm) of mammary tissue taken from a transgenic mouse (f) or a normal littermate mouse (e) stained with hematoxylin and eosin.

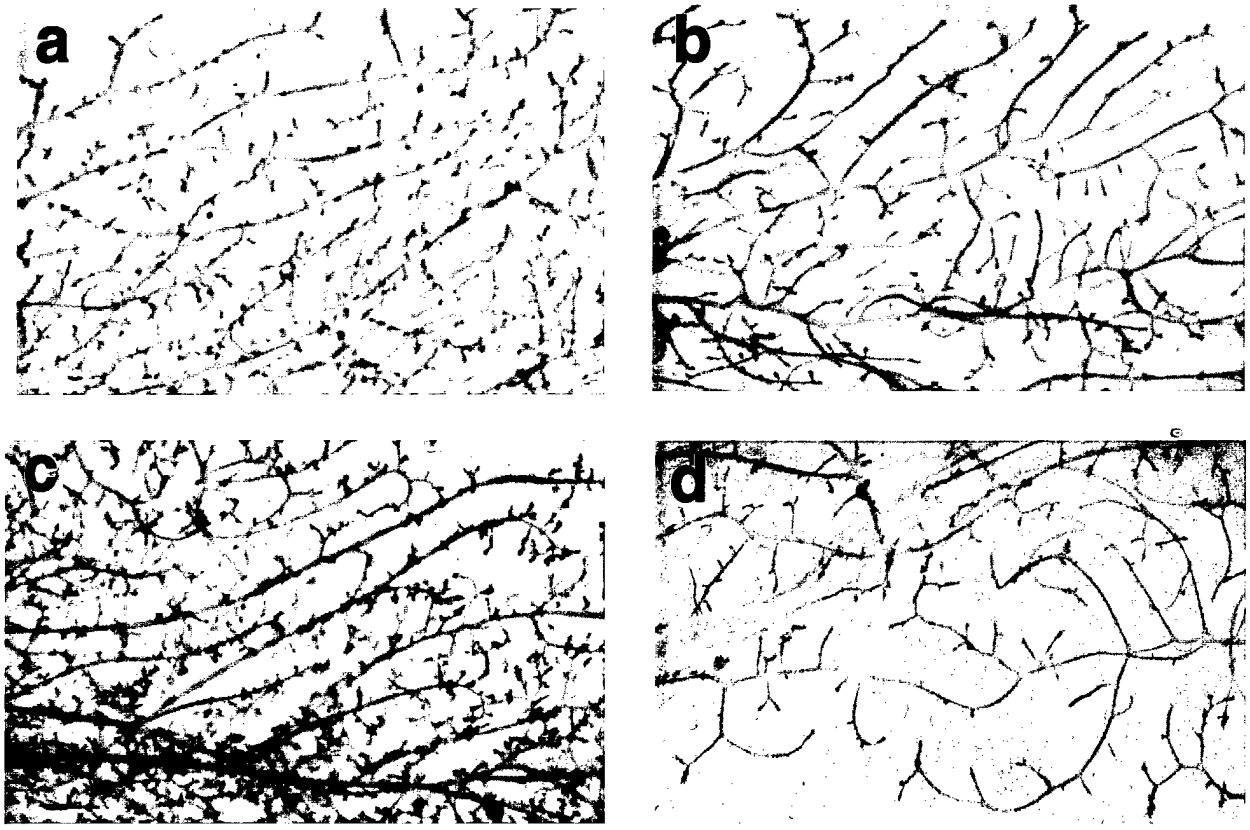

Figure 5. Whole-mount analysis of mammary glands from singly and doubly transgenic mice. The 4th inguinal mammary glands were taken from 6 month old virgin mice of a normal littermate (a), an AP-2α singly transgenic mouse (b), a neu singly transgenic mouse (c) and an AP-2α/neu doubly transgenic mouse (d).